PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 12 May 2000 (12.05.00)	
International application No. PCT/US99/17806	**Applicant's or agent's file reference** RUT.00-0010
International filing date (day/month/year) 03 August 1999 (03.08.99)	**Priority date** (day/month/year) 03 August 1998 (03.08.98)
Applicant MALIGA, Pal et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

02 March 2000 (02.03.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Claudio Borton
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

From the INTERNATIONAL BUREAU

To:

RIGAUT, Kathleen, D.
Dann, Dorfman, Herrell and Skillman
Suite 720
1601 Market Street
Philadelphia, PA 19103
ETATS-UNIS D'AMERIQUE

NOTIFICATION CONCERNING SUBMISSION OR TRANSMITTAL OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

Date of mailing (day/month/year) 06 April 2000 (06.04.00)	
Applicant's or agent's file reference RUT.00-0010	**IMPORTANT NOTIFICATION**
International application No. PCT/US99/17806	International filing date (day/month/year) 03 August 1999 (03.08.99)
International publication date (day/month/year) 17 February 2000 (17.02.00)	Priority date (day/month/year) 03 August 1998 (03.08.98)
Applicant RUTGERS, THE STATE UNIVERSITY OF NEW JERSEY et al	

1. The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).

2. This updates and replaces any previously issued notification concerning submission or transmittal of priority documents.

3. An **asterisk(*)** appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

4. The **letters "NR"** appearing in the right-hand column denote a priority document which was not received by the International Bureau or which the applicant did not request the receiving Office to prepare and transmit to the International Bureau, as provided by Rule 17.1(a) or (b), respectively. In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
03 Augu 1998 (03.08.98)	60/095,163	US	28 Marc 2000 (28.03.00)
03 Augu 1998 (03.08.98)	60/095,167	US	30 Sept 1999 (30.09.99)
15 Dece 1998 (15.12.98)	60/112,257	US	30 Sept 1999 (30.09.99)
29 Apri 1999 (29.04.99)	60/131,611	US	30 Sept 1999 (30.09.99)
11 June 1999 (11.06.99)	60/138,764	US	30 Sept 1999 (30.09.99)

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer

Beatriz Morariu

Telephone No. (41-22) 338.83.38

WO 00/07431
PCT/US99/17806

PCT

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

From the INTERNATIONAL BUREAU

To:
RIGAUT, Kathleen, D.
Dann, Dorfman, Herrell and Skillman
Suite 720
1601 Market Street
Philadelphia, PA 19103
ÉTATS-UNIS D'AMÉRIQUE

Date of mailing (day/month/year) 17 February 2000 (17.02.00)	
Applicant's or agent's file reference RUT.00-0010	**IMPORTANT NOTICE**

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/US99/17806	03 August 1999 (03.08.99)	03 August 1998 (03.08.98)

Applicant
RUTGERS, THE STATE UNIVERSITY OF NEW JERSEY et al

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AU,CN,EP,IL,JP,KP,KR,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   AE,AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,CA,CH,CR,CU,CZ,DE,DK,EA,EE,ES,FI,GB,GD,GE,GH,GM,HR,HU,ID,IN,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MD,MG,MK,MN,MW,MX,NO,NZ,OA,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,UA,UG,UZ,VN,YU,ZA,ZW
   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   17 February 2000 (17.02.00) under No. WO 00/07431

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, **a demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer
J. Zahra

Telephone No. (41-22) 338.83.38

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/17806

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01H 4/00, 5/00; C12N 15/29, 15/31, 15/65, 15/67, 15/82, 15/84

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EMBL, Genbank

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 97/06250 A1 (RUTGERS UNIVERSITY) 20 February 1997, pages 1, 3-6, 12-14, 17-18 and Figures 1 and 5.	1-6 and 15-17
Y	NIELSEN et al. Characterization of the Pea Chloroplast DNA *Ori*A Region. Plasmid. 1993, Vol. 30, pages 197-211, see page 197.	1-6
Y	SEKIYA et al. Sequence of the gene for isoleucine $tRNA_1$ and the surrounding region in a ribosomal RNA operon of Escherichia coli. Nucleic Acids Research. 1979, Vol. 6, pages 575-592, see page 575.	1-6

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 07 NOVEMBER 1999	Date of mailing of the international search report 30 NOV 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DAVID T. FOX Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/17806

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SPRENGART et al. The downstream box: an efficient and independent translation intitiation signal in *Escherichia coli*. The EMBO Journal. 1996, Vol. 15, No. 3, pages 665-674, see page 665.	1-6
Y	VASIL et al. Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus. Bio/Technology. June 1992, Vol. 10, No. 6, pages 667-674, see page 667.	7-8 and 18-26
Y	PANG et al. An Improved Green Fluorescent Protein Gene as a Vital Marker in Plants. Plant Physiology. 1996, Vol. 112, pages 893-900, see page 893.	9-14
Y	HIRATSUKA et al. The complete sequence of the rice (*Oryza sativa*) chloroplast genome: Intermolecular recombination between distinct tRNA genes accounts for a major plastid DNA inversion during the evolution of cereals. Molecular and General Genetics. 1989, Vol. 217, pages 185-194, see page 185.	18-26
Y	ZHANG et al. Efficient regeneration of transgenic plants from rice protoplasts and correctly regulated expression of the foreign gene in plants. Theoretical and Applied Genetics. 1988, Vol. 76, pages 835-840, see page 835.	18-23
Y	THOMPSON et al. Protoplast culture of rice (Oryza sativa L.) using media solidified with *agarose*. Plant Science. 1986, Vol. 47, No. 2, pages 123-133, see page 123.	24-26
Y	MAKRIDES, S.C. Strategies for Achieving High-Level Expression of Genes in *Escherichia coli*. Microbiological Reviews. September 1996, Vol. 60, No. 3, pages 512-538, see page 524.	27-28
Y	MURRAY et al. Codon usage in plant genes. Nucleic Acids Research. 1989, Vol. 17, No. 2, pages 477-498, see pages 477 and 491.	27-28

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/17806

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/17806

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/69.7, 320.1, 424, 430, 430.1, 431, 468, 469, 470; 536/23.6, 23.7, 24.1; 800/278, 287, 288, 292, 293, 294, 300, 300.1, 320, 320.1, 320.2, 320.3

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

435/69.7, 320.1, 424, 430, 430.1, 431, 468, 469, 470; 536/23.6, 23.7, 24.1; 800/278, 287, 288, 292, 293, 294, 300, 300.1, 320, 320.1, 320.2, 320.3

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-26, drawn to a DNA construct comprising a promoter element, a leader, and a downstream box element, and a method for its use for enhanced plastid expression of a heterologous gene.

Group II, claim(s) 27-28, drawn to a method for modifying codon usage in structural genes.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The inventions of each of Groups I, drawn to a first product and method of use, and Group II, drawn to a second method, are taught or suggested by the prior art, as outlined in the accompanying Search Report, and so each do not constitute an advance over the prior art which would constitute a special technical feature. Furthermore, the various leader sequences and downstream box elements of Group I, and the methods for plastid transformation of Group I, are not required by Group II; while the methods of structural gene modification of Group II are not required by Group I. Accordingly, the inventions do not relate to a single inventive concept which would constitute a single special technical feature.

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PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

14

Applicant's or agent's file reference RUT.00-0010	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US99/17806	International filing date *(day/month/year)* 03 AUGUST 1999	Priority date *(day/month/year)* 03 AUGUST 1998
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant RUTGERS, THE STATE UNIVERSITY OF NEW JERSEY		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 9 sheets.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability
   - IV ☒ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
02 MARCH 2000	05 FEBRUARY 2001
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DAVID T. FOX Telephone No. (703) 308-0196

TERRY J. DEY
PARALEGAL SPECIALIST
TECHNOLOGY CENTER 1600

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US99/17806

I. Basis of the report

1. With regard to the **elements** of the international application:*

[X] the international application as originally filed

[X] the description:
pages 1-103 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the claims:
pages 104-109 , as originally filed
pages NONE , as amended (together with any statement) under Article 19
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the drawings:
pages 1-49 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the sequence listing part of the description:
pages 1-26 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

[X] contained in the international application in printed form.

[X] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. [X] The amendments have resulted in the cancellation of:

[X] the description, pages NONE

[X] the claims, Nos. NONE

[X] the drawings, sheets/~~fig~~ NONE

5. [X] This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

***Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

- [ ] restricted the claims.
- [X] paid additional fees.
- [ ] paid additional fees under protest.
- [ ] neither restricted nor paid additional fees.

2. [ ] This Authority found that the requirement of unity of invention is not complied with and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

- [ ] complied with.
- [X] not complied with for the following reasons:

Please See Supplemental Sheet.

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

- [X] all parts.
- [ ] the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)	Claims	3-4 and 6-28	YES
	Claims	1-2 and 5	NO
Inventive Step (IS)	Claims	NONE	YES
	Claims	1-28	NO
Industrial Applicability (IA)	Claims	1-28	YES
	Claims	NONE	NO

2. citations and explanations (Rule 70.7)

Claims 3-4 and 6-28 meet the criteria set out in PCT Articles 33(2) and (3), because the prior art does not teach chloroplast expression vectors comprising heterolgous promoter elements, leaders, and downstream box elements, wherein such vectors have the industrial applicability of introducing agronomic traits into crop plants.

Claims 1-2 and 5 lack novelty under PCT Article 33(2) as being anticipated by Zoubenko et al.

Zoubenko et al teach a chloroplast expression vector comprising a recombinant construct comprising an rrn or psbA promoter and a heterologous aadA gene encoding a spectinomycin resistance protein (see, e.g., page 3820, column 1, second and third full paragraphs), wherein the promoters and/or constructs would inherently comprise the native leader sequence and downstream box element present in either the promoters or the structural gene.

Claims 1-5 and 15-17 lack an inventive step under PCT Article 33(3) as being obvious over Zoubenko et al in view of Sprengart et al.

Zoubenko et al teach a chloroplast expression vector as discussed above, wherein the vector also comprises chloroplast termination regions and regions of chloroplast homology for homologous recombination (see, e.g., page 3821, Figures 1 and 2), and also teach biolistic transformation of tobacco cells and regeneration of whole transplastomic plants bearing seed (see, e.g., page 3819, Abstract), but do not explicitly teach the particularly claimed combinations of heterologous promoters, leaders, and downstream box elements.

Sprengart et al teach the importance of the downstream box element in gene expression (see, e.g., page 665, Abstract; page 666, Figure 1A; page 667, column 2, bottom paragraph).

It would have been obvious to one of ordinary skill in the art to utilize the chloroplast expression vector comprising known

(Continued on Supplemental Sheet.)

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

Claim 4 is objected to under PCT Rule 66.2(a)(iii) as containing the following defect(s) in the form or contents thereof : The recitation of "15" at the end of the claim should be "16".

Claim 8 is objected to under PCT Rule 66.2(a)(iii) as containing the following defect(s) in the form or contents thereof : The phrase --the sequence-- should be inserted after "having" in line 4.

Claim 21 is objected to under PCT Rule 66.2(a)(iii) as containing the following defect(s) in the form or contents thereof: The claim is missing a period at the end.

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

Claims 2 and 5 are objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 6 because the claims are indefinite for the following reason(s): The claims are duplicates.

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

CLASSIFICATION:
The International Patent Classification (IPC) and/or the National classification are as listed below:
IPC(7): A01H 4/00, 5/00; C12N 15/29, 15/31, 15/65, 15/67, 15/82, 15/84 and US Cl.: 435/69.7, 320.1, 424, 430, 430.1, 431, 468, 469, 470; 536/23,6, 23.7, 24.1; 800/278, 287, 288, 292, 293, 294, 300, 300.1, 320, 320.1, 320.2, 320.3

I. BASIS OF REPORT:

5. (Some) amendments are considered to go beyond the disclosure as filed:
NONE

IV. LACK OF UNITY OF INVENTION:

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2, and 13.3 is not complied with for the following reasons:
As applicant was previously notified this International Preliminary Examining Authority has found plural inventions claimed in the International Application covered by the claims indicated below:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-26, drawn to a DNA construct comprising a promoter element, a leader, and a downstream box element, and a method for its use for enhanced plastid expression of a heterologous gene.

Group II, claim(s) 27-28, drawn to a method for modifying codon usage in structural genes.

and it considers that the International Application does not comply with the requirements of unity of invention (Rules 13.1, 13.2 and 13.3) for the reasons indicated below:

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The inventions of each of Groups I, drawn to a first product and method of use, and Group II, drawn to a second method, are taught or suggested by the prior art, as outlined in the accompanying Search Report, and so each do not constitute an advance over the prior art which would constitute a special technical feature. Furthermore, the various leader sequences and downstream box elements of Group I, and the methods for plastid transformation of Group I, are not required by Group II; while the methods of structural gene modification of Group II are not required by Group I. Accordingly, the inventions do not relate to a single inventive concept which would constitute a single special technical feature.

V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):
chloroplast promoters, terminators, leaders, regions of homology, and antibiotic resistance genes as taught by Zoubenko et al, and to modify that method by incorporating specific leader sequences and downstream box elements, given their importance as taught by Sprengart et al and the knowledge by those of ordinary skill in the art of the similarity between chloroplast and prokaryotic gene regulation and expression. Choice of particular promoter, leader, or downstream box element would have been the optimization of process parameters.

Claim 6 lacks an inventive step under PCT Article 33(3) as being obvious over the prior art as applied in the immediately preceding paragraph and further in view of each of Nielsen et al or Sekiya et al.

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

Zoubenko et al in view of Sprengart et al teach a construct comprising a chloroplast promoter, leader and downstream box element as discussed above, but do not teach the particularly claimed downstream box elements.

Each of Nielsen et al or Sekiya et al teach the claimed sequences (see, e.g., Nielsen et al, page 197, Abstract; page 205, and attached Sequence Search results; and Sekiya et al, page 575, Abstract; pages 583-585, Figures 5-7; and attached Sequence Search results).

It would have been obvious to one of ordinary skill in the art to utilize the DNA construct for chloroplast transformation as taught by Zoubenko et al in view of Sprengart et al, and to modify that construct by incorporating the particular downstream box elements taught by each of Nielsen et al or Sekiya et al, given the recognition by those of ordinary skill in the art that choice of downstream box element would have been the optimization of process parameters.

Claim 7 lacks an inventive step under PCT Article 33(3) as being obvious over Zoubenko et al in view of Sprengart et al, further in view of Vasil et al.

Zoubenko et al in view of Sprengart et al teach a construct comprising a promoter, leader, downstream box element, and spectinomycin resistance gene for chloroplast transformation as discussed above, but do not teach the use of the bar gene encoding phosphinothricin resistance.

Vasil et al teach the use of the bar gene encoding phosphinothricin resistance as a selectable marker gene for biolistically-transformed plants (see, e.g., page 667, Abstract).

It would have been obvious to one of ordinary skill in the art to utilize the method of plastid transformation taught by Zoubenko et al in view of Sprengart et al, and to modify the construct by incorporating the bar gene taught by Vasil et al, given the recognition by those of ordinary skill in the art that choice of selectable marker gene would have been the optimization of process parameters.

Claim 8 lacks an inventive step under PCT Article 33(3) as being obvious over the prior art as applied in the immediately preceding paragraph and further in view of Murray et al and Makrides.

Zoubenko in view of Sprengart et al, further in view of Vasil et al, teach a construct comprising a chloroplast promoter, leader, bar gene, and downstream element as discussed above, but do not teach the use of a synthetic bar gene sequence.

Murray et al teach the determination of codon optimization for particular hosts including plants prior to transformation with microbial genes, wherein the arginine codons AGA and AGG were predominant in plants (see, e.g., page 477, Abstract; page 478, last sentence of top paragraph; pages 483-484; page 491, second full paragraph).

Makrides teaches that microbes such as E. coli rarely use AGA or AGG codons for arginine (see, e.g., page 524, Table 4).

It would have been obvious to one of ordinary skill in the art to utilize the construct taught by Zoubenko et al in view of Sprengart et al, further in view of Vasil et al, and to modify that construct by modifying the codon usage of the bar gene to favor plant expression, as taught by Murray et al in view of Makrides, as suggested by Murray et al.

Claims 27-28 lack an inventive step under PCT Article 33(3) as being obvious over Murray et al in view of Makrides, further in view of Vasil et al.

Murray et al teach a method for determining optimized codon usage in plants for genetic engineering, including those codons favored for arginine, and suggest modification thereof to favor plants and discourage microbial expression as discussed above, but do not teach the incorporation of rare microbial codons or the use of the bar gene.

Makrides teaches that AGA and AGG are rarely used as arginine codons in microbes, as discussed above.

Vasil et al teach the use of the bar gene in plant transformation as discussed above.

It would have been obvious to one of ordinary skill in the art to utilize the method of codon optimization for plant expression taught by Murray et al, and to modify that method by incorporating the particular rare arginine codons taught by Makrides and the particular gene taught by Vasil et al, as suggested by Murray et al, given the recognition by those of ordinary skill in the art that choice of gene for plant expression would have been the optimization of process parameters.

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

Claims 9-14 lack an inventive step under PCT Article 33(3) as being obvious over Zoubenko et al in view of Sprengart et al, further in view of Pang et al.

Zoubenko et al in view of Sprengart et al teach a construct for chloroplast transformation as discussed above, but do not teach the use of a fusion protein comprising a fluorescent protein.

Pang et al teach the use of the green fluorescent protein gene as a useful marker gene for transformed corn and tobacco cells, and suggest the use of fusion protein-encoding genes (see, e.g., page 893, Abstract and column 1, bottom paragraph; page 899, column 1).

It would have been obvious to one of ordinary skill in the art to utilize the construct for chloroplast transformation taught by Zoubenko et al taken with Sprengart et al, and to modify that construct by incorporating the gfp gene taught by Pang et al as a fusion protein-encoding gene, as suggested by Pang et al.

Claims 18-23 lack an inventive step under PCT Article 33(3) as being obvious over Zoubenko et al in view of Vasil et al, further in view of Maliga et al.

Zoubenko et al teach a method for producing transplastomic plants comprising biolistic transformation with a construct encoding a selectable antibiotic resistance marker, applying said antibiotic to said cells to select for transformed plastids, and regenerating whole plants from the surviving cells as discussed above, but do not teach the application to monocots.

Vasil et al teach monocot transformation via biolistics and whole plant regeneration as discussed above.

Maliga et al teach the advantages of plastid transformation for the introduction of desired heterologous genes and suggest the broad application of the technique (see, e.g., paragraph bridging pages 207 and 208; page 208, first full paragraph).

It would have been obvious to one of ordinary skill in the art to utilize the method of chloroplast transformation taught by Zoubenko et al, and to modify that method by incorporating monocots, as suggested by Maliga et al.

Claims 24-26 lack an inventive step under PCT Article 33(3) as being obvious over Zoubenko et al in view of Zhang et al, further in view of Thompson et al, further in view of Maliga et al.

Zoubenko et al teach a microprojectile-mediated method of chloroplast transformation and whole plant regeneration as discussed above, but do not teach the use of rice.

Zhang et al teach a method for rice transformation and regeneration (see, e.g., page 835, Abstract).

Thompson et al teach the use of AA medium in rice tissue culture (see, e.g., page 123, Abstract and column 2, first full paragraph).

Maliga et al suggest the application of chloroplast transformation to a variety of crops as discussed above.

It would have been obvious to one of ordinary skill in the art to utilize the method of chloroplast transformation and whole plant regeneration taught by Zoubenko et al, and to modify that method by incorporating rice transformation as taught by Zhang et al and rice culture media as taught by Thompson et al, given the suggestion to do so by Zhang et al and Maliga et al, and the recognition by those of ordinary skill in the art that choice of known rice tissue culture media would have been the optimization of process parameters.

---------- NEW CITATIONS ----------

ZOUBENKO et al. Efficient targeting of foreign genes into the tobacco plastid genome. Nucleic Acids Research. 1994, Vol. 22, No. 19, pages 3819-3824, see page 3820.

MALIGA et al. Plastid engineering in land plants: a conservative genome is open to change. Philosophical Transactions of the Royal Society of London, B. 1993, Vol. 342, pages 203-208, see pages 207-208.

☐1: X05822. Streptomyces hygr...[gi:47126] Links

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LOCUS       SHBAR                     835 bp    DNA     linear   BCT 10-FEB-1999
DEFINITION  Streptomyces hygroscopicus bar gene conferring resistance to
            herbicide bialaphos.
ACCESSION   X05822
VERSION     X05822.1  GI:47126
KEYWORDS    bar gene; herbicide resistance; phosmothicin acetyl transferase.
SOURCE      Streptomyces hygroscopicus
  ORGANISM  Streptomyces hygroscopicus
            Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;
            Streptomycineae; Streptomycetaceae; Streptomyces.
REFERENCE   1  (bases 1 to 835)
  AUTHORS   Thompson,C.J., Movva,N.R., Tizard,R., Crameri,R., Davies,J.E.,
            Lauwereys,M. and Botterman,J.
  TITLE     Characterization of the herbicide-resistance gene bar from
            Streptomyces hygroscopicus
  JOURNAL   EMBO J. 6, 2519-2523 (1987)
COMMENT     The bar gene contains six possible reading frames. The most likely
            ORF (see feature table) containing the codons used in streptomycete
            structural genes codes for a 21 kd protein.
            Data kindly reviewed (12-OCT-1987) by BOTTERMAN J.
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                     VGFWQLDFSLPVPPRPVLPVTEI"
BASE COUNT      158 a    283 c    274 g    120 t
ORIGIN
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       61 actcgacgac gcgtaaaacg atcgaccacg tacacgagtc cggacacggg gcgaggaggc
      121 ccggttccgg caccgaggaa gaccgaagga agaccacacg tgagcccaga acgacgcccg
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      781 cgcaatggtg aggtggaacc gcggcggatc gcgtacgacc gtgcccagga ggcct
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Herbicide resistance
Agr gne transf

Engineering herbicide resistance in plants by expression of a detoxifying enzyme

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Communicated by M.Van Montagu

Phosphinothricin (PPT) is a potent inhibitor of glutamine synthetase in plants and is used as a non-selective herbicide. The *bar* gene which confers resistance in *Streptomyces hygroscopicus* to bialaphos, a tripeptide containing PPT, encodes a phosphinothricin acetyltransferase (PAT) (see accompanying paper). The *bar* gene was placed under control of the 35S promoter of the cauliflower mosaic virus and transferred to plant cells using *Agrobacterium*-mediated transformation. PAT was used as a selectable marker in protoplast co-cultivation. The chimeric *bar* gene was expressed in tobacco, potato and tomato plants. Transgenic plants showed complete resistance towards high doses of the commercial formulations of phosphinothricin and bialaphos. These data present a successful approach to obtain herbicide-resistant plants by detoxification of the herbicide.

Key words: acetyltranferase/bialaphos/herbicide resistance/glutamine synthetase/phosphinothricin

Introduction

The use of herbicides to reduce loss in crop yield due to weeds has become an integral part of modern agriculture. There is a continuous search for new herbicides that are highly effective and safe for animals and the environment. A new class of herbicides that fulfils these needs acts by inhibiting specific amino acid biosynthesis pathways in plants (La Rossa and Falco, 1984). However, most of these herbicides do not distinguish between weeds and crops.

Modifying plants to become resistant to such broad-spectrum herbicides would allow their selective use for crop protection. As a consequence, a major effort has been devoted in several laboratories to engineer herbicide-resistant plants. Two approaches have been followed. In the first, a mutant form of the target enzyme is produced which is still active but less sensitive to the herbicide. In this way, mutant plants producing an altered form of the enzyme acetolactate synthase have been selected which are resistant to the sulfonylurea and imidazolinone herbicides (Chaleff and Ray, 1984; Shaner and Anderson, 1985). In another example, a mutant form of the bacterial *aro*A gene was expressed in tobacco and conferred tolerance to the herbicide glyphosate (Comai *et al.*, 1985). The second approach involves overproduction of the target enzyme. It has been demonstrated that overexpression of the plant enzyme 5-enol-pyruvylshikimate-3 phosphate synthase conferred glyphosate tolerance in transgenic petunia plants (Shah *et al.*, 1986).

Bialaphos (Ogawa *et al.*, 1973) and phosphinothricin (PPT) (Bayer *et al.*, 1972) are potent new herbicides. Bialaphos is a tripeptide antibiotic produced by *Streptomyces hygroscopicus*. It consists of PPT, an analogue of L-glutamic acid, and two L-alanine residues. Upon removal of these residues by peptidases, PPT is a potent inhibitor of glutamine synthetase (GS). This enzyme plays a central role in the assimilation of ammonia and in the regulation of nitrogen metabolism in plants (Miflin and Lea, 1977; Skokut *et al.*, 1978). It is the only enzyme in plants that can detoxify ammonia released by nitrate reduction, amino acid degradation and photorespiration. Inhibition of GS by PPT causes rapid accumulation of ammonia which leads to death of the plant cell (Tachibana *et al.*, 1986). PPT is chemically synthesized (Basta®, Hoechst AG) while bialaphos is produced by fermentation of *S. hygroscopicus* (Herbiace®, Meiji Seika Ltd). Here we present an alternative strategy to engineer herbicide resistance in plants by expressing an enzyme that detoxifies phosphinothricin.

The accompanying paper describes the cloning and characterization of a bialaphos resistance gene (*bar*) from *S. hygroscopicus* which is involved in the bialaphos biosynthesis pathway. It encodes a phosphinothricin acetyltransferase (PAT), which acetylates the free NH_2 group of PPT and thereby prevents autotoxicity in the producing organism (Murakami *et al.*, 1986). Here we report the expression of the *bar* gene in transgenic tobacco, tomato and potato plants. We show that transgenic plants expressing PAT are completely resistant to high doses of the commercial formulations of both PPT and bialaphos. The use of the *bar* gene as reporter gene to analyse plant gene expression is also demonstrated.

Results

Chimeric bar gene for expression in plants

The *bar* gene was originally isolated in a streptomycete vector and subcloned into an *Escherichia coli* vector yielding pBG195 (accompanying paper). The translation initiation codon of the *bar* gene in *Streptomyces* is GTG. In order to guarantee proper translation initiation in plants, an ATG initiation codon is required. To this end, the N-terminal end of the *bar* coding region was substituted for two complementary synthetic oligonucleotides (Figure 1A). In the resulting plasmid, pGSFR1, the *bar* gene contains an ATG as initiation codon. Subsequently, this gene was inserted between the cauliflower mosaic 35S promoter and the termination and polyadenylation signal of the octopine T-DNA gene 7 (Velten and Schell, 1985). This chimeric gene and a kanamycin resistance gene under the control of the nopaline synthase promoter were inserted between the octopine T-DNA borders of plant transformation vector pGV1500 (Figure 1B). This plasmid, pGSFR280, was mobilized into the *Agrobacterium* recipient C58C1 Rif^R (pGV2260) (Deblaere *et al.*, 1985), to generate strain C58C1 Rif^R (pGSFR1280).

PAT is a selectable marker in tobacco protoplast transformation

To test whether expression of the PAT enzyme allows for selec-

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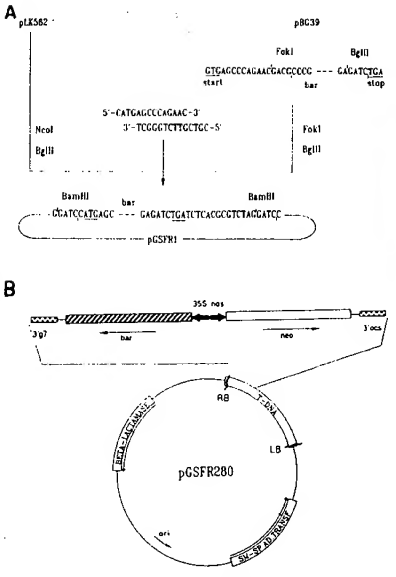

Fig. 1. (A) Construction of a *bar* gene cassette. pGSFR1 contains an intact *bar* gene with an ATG initiation codon. The nucleotide sequence of the 5' and 3' end of the gene are shown and the initiation and stop codon are underlined. (B) Schematic representation of pGSFR280. Both chimeric genes, the *bar* gene under control of the CaMV 35S promoter and the *neo* gene under control of the *nos* promoter, are inserted in divergent orientation between the T-DNA border repeats of pGV1500 (RB: right border and LB: left border). The respective genes are followed by fragments encoding termination and polyadenylation signals of ocs (3'ocs) and T-DNA gene 7 (3'g7).

tion of transformed plant cells, leaf protoplasts of *Nicotiana tabacum* cv. Petit Havana (SR1) were co-cultivated with the *Agrobacterium* strains C58C1 Rif^R (pGSFR1280). C58C1 Rif^R (pGV2260) was used as a control strain that lacks the *bar* gene. Transformants were selected in medium containing 50 mg/l kanamycin or various concentrations of PPT (0.5–50 mg/l). After 1 month, no growth was observed on the selective plates of the C58C1 Rif^R (pGV2260) co-cultivation. Thus, the low dose of 0.5 mg/l PPT is sufficient to inhibit growth of sensitive cells. Calli transformed with C58C1 Rif^R (pGSFR1280) grew indistinguishable from their growth in non-selective medium under all selective conditions. Twenty calli that had been selected on 50 mg/l PPT were then grown for 2 months on medium containing 50 mg/l PPT. Subsequently they were subcultured on media containing different levels of PPT ranging from 50 to 1000 mg/l. All calli grew on medium containing 500 mg/l PPT. At a concentration of 1000 mg/l of PPT, their growth rate was slightly reduced, and three out of the 20 calli died after a few weeks.

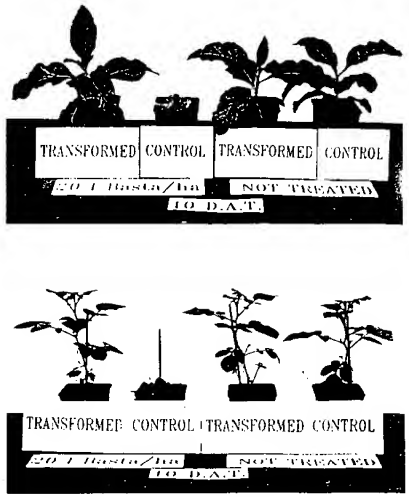

Fig. 2. Herbicide resistance in transgenic tobacco plants (*N. tabacum* cv. Petit Havana SR1 plants) (A) and transgenic potato plants (*S. tuberosum* cv. Berolina). (B) Untransformed control and transformed plants were sprayed with 20 l Basta® /ha. A control series of plants was not treated. The treated control plant was killed within 10 days, while the treated transformed plant grew as well as the untreated control.

Transgenic plants are fully resistant to the herbicides Basta® and Herbiace®

N. tabacum cv. Petit Havana SR1 plants expressing the *bar* gene were obtained by the leaf disc infection method (Horsch *et al.* 1985). Transformed shoots were selected after infection with C58C1Rif^R (pGSFR1280) on medium containing either 50 mg/l kanamycin or 1–50 mg/l PPT. Leaf discs infected with control strain C58C1Rif^R (pGV2260) bleached within 1 week on PPT-containing medium.

We then evaluated whether expression of PAT confers resistance to commercial formulations of PPT and bialaphos. Transgenic plants were transferred to soil and grown in the greenhouse. Their growth was indistinguishable from untransformed control plants. Control SR1 and transgenic plants were sprayed with doses equivalent to 8 l/ha and 20 l/ha Basta®. Basta® at 2 l/ha effectively kills control SR1 plants in 10 days. All 21 of the transgenic plants which were assayed were fully resistant to the herbicide (Figure 2). Two additional applications of the herbicide within a 4-week period did not affect growth of the plants. Treated plants flowered normally and set seed. Transgenic plants were also sprayed with 8 l/ha and 20 l/ha of Herbiace®, the commercial formulation of bialaphos. They also proved to be fully resistant to these applications.

Ammonia levels in herbicide-treated plants

As a more sensitive indicator of GS inhibition, we have measured

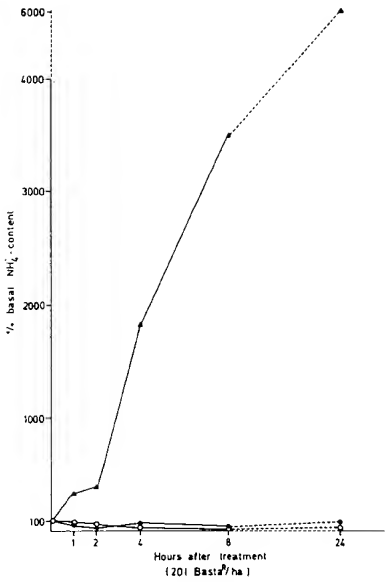

Fig. 3. Ammonia determination (% basal NH_4^+ content) in transformed and untransformed tobacco plants after spraying with 20 l Basta® /ha. Samples were taken before spraying and at regular time points after spraying. ○ untreated control plant; ● treated transgenic plant; ▲ treated control plant.

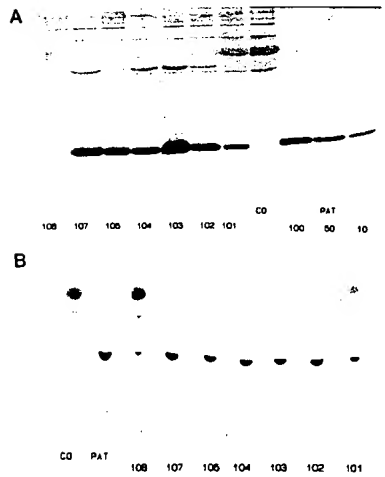

Fig. 4. (A) Detection of PAT protein by immunoblotting of leaf extracts from transgenic *N. tabacum* plants. Crude extracts of 50 μg protein were separated on an SDS–polyacrylamide gel and electrophoretically transferred to nitrocellulose. The filter was treated with PAT antiserum and stained. **Lanes 1–7**, crude extracts from seven independent plants. **Lane 8** crude extract from an untransformed plant (CO). **Lanes 9–11**, 100, 50 and 10 ng of purified PAT. (B) Detection of PAT activity by t.l.c. The extracts were prepared from leaf tissue and diluted 100 times. The reaction was performed and an aliquot spotted on a silicagel t.l.c. plate for ascendent chromatography. [^{14}C]Substrates and reaction products were visualized by autoradiography. **Lane 1**, leaf extract from untransformed plant. **Lane 2**, leaf extract of untransformed plant to which PAT enzyme is added. **Lanes 3–9**, extracts from seven independent transgenic tobacco plants.

ammonia accumulation in transgenic and non-transformed plants treated with the herbicide. Ammonia accumulated rapidly in treated control plants and increased 40-fold after 8 h. Ammonia levels in transgenic plants did not significantly change over a 24-h period after application of 20 l Basta®/ha. The levels were comparable to those present in untreated SR1 plants (Figure 3). This clearly shows that the transgenic plant is completely insensitive to the herbicide treatment.

Inheritance of PPT resistance

Seven transgenic plants that had been treated with 20 l/ha Basta® were analysed further (plants 101–105, 107 and 108). They produced normal amounts of viable seed. Five week old plants from the F1 progeny were sprayed with 8 l/ha Basta®. From the seven parental plants, six segregated resistant and sensitive seedlings in a 3 to 1 ratio, indicating Mendelian inheritance as a single dominant locus. Southern blots showed that 103 contained three copies of *bar*, whereas the others carried a single copy (data not shown). Since a Mendelian inheritance was observed, we expect that the three T-DNA copies are inserted at a single locus in plant 103. One plant (101) did not produce resistant seedlings. Also no PAT enzyme activity could be detected in these seedlings. This observation needs further investigation.

Expression of PAT in transgenic plants

The expression level of PAT was determined in the seven selected transformed tobacco plants. Analysis of crude leaf extracts by Western blotting revealed the synthesis of a polypeptide that co-migrated with PAT, purified from an *E. coli* overproducing strain (in preparation) (Figure 4A). Plants 101 and 108 produce low levels of PAT (<0.01%), whereas it amounts up to 0.1% of total soluble protein in the others. The same relative levels of PAT activity were observed in an enzymatic assay in which ^{14}C-labelled acetylated PPT was detected after separation by t.l.c. (Figure 4B). Using a spectrophotometric assay which is more convenient for quantification, no activity above the background level could be measured in extracts of 108, whereas a 100-fold dilution of the extract of 103 still contained detectable activity (Table I). Interestingly, the low level expression in 108 was still sufficient to fully protect the plant from the herbicide.

PAT-specific mRNA was detected in a Northern blot of total RNA extracted from young leaves of the plants (Figure 5). Although all of these plants contain the same gene, they showed at least a 100-fold variation in the accumulation of PAT-specific mRNA as assayed by autoradiography densitometry. In the different plants the ratio in the levels of steady-state mRNA corre-

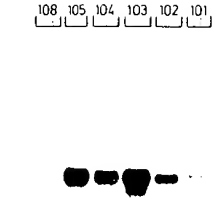

Fig. 5. Northern blot hybridization of RNA extracted from six transgenic tobacco plants. 10 μg aliquots of total RNA were electrophoresed in 1.5% agarose/formaldehyde gel and blotted to nitrocellulose. A *bar* RNA probe was used for hybridization.

Table I. PAT activity in crude leaf extracts in transgenic tobacco plants measured spectrophotometrically

Plants	Activity (10^{-6} units/mg extracted protein)
108	n.d.
107	704
105	264
104	304
103	1240
102	407
101	43

Enzyme assay and definition of unit activity are described in Materials and methods. n.d. = not detectable

sponded to the amount of PAT protein accumulated (compare Figure 5 and Figure 4A).

Herbicide-resistant tomato and potato plants

Transgenic plants expressing PAT were obtained from leaf disc infections of *Lycopersicum esculentum* cv. Lukullus and *Solanum tuberosum* cv. Berolina, cv. Bintje and cv. Désiree. The two tomato and three potato plants, which were sprayed with 20 l/ha Basta®, were fully resistant (Figure 2B). All the transgenic plants tested (15 tomatoes and 26 potatoes) showed high levels of PAT activity.

Discussion

The *bar* gene from *S. hygroscopicus* encodes a PAT which converts PPT into the non-toxic acetylated form (accompanying paper). We have used *Agrobacterium* vectors to transfer the *bar* gene in tobacco, tomato and potato plants. When expressed from the constitutive cauliflower mosaic virus 35S promoter an active PAT enzyme was produced in the plant cells. Transgenic plants were completely resistant towards the herbicide. We also demonstrated the use of *bar* gene as a convenient selectable and screenable marker in plant transformation experiments.

Low doses of 0.5 mg/l PPT are sufficient to inhibit growth of tobacco protoplasts. The *bar* gene proved to be an efficient dominant selectable marker in tobacco protoplast transformation. Tobacco calli expressing the PAT enzyme grew on medium containing 500 mg/l PPT. It was also possible to select directly for transformed shoots in leaf disc transformations. Similar results were obtained with chimeric constructs carrying the *bar* gene under control of other plant promoters (unpublished data).

The *bar* gene is a useful reporter gene. It is enzymatically and immunologically assayable to analyse gene expression in transformed plants. In Western blots, amounts of 5 ng PAT were detectable (Figure 3A). In addition, its enzymatic activity can be analysed by chromatographic detection of acetylated PPT (Figure 3B). Enzymatic activities corresponding to 1 ng of protein are clearly observed after an overnight exposure. Accurate values of enzyme activity are obtained if the enzyme kinetics are analysed spectrophotometrically (see Table I). Enzyme concentrations of 5 ng/ml can be detected above background acetylase activity in plant extracts. Enzymatic characterization of PAT and some derivatives will be described elsewhere.

Analysis of the PAT expression demonstrated that there is a substantial variation between independent transformants in the levels of expression, as has been observed in other cases (Jones *et al.*, 1985; Sanders *et al.*, 1987). Quantitative data of activities showed at least a 30-fold difference. This variability between different plants was represented at the level of PAT synthesis, PAT activity and the amount of PAT-specific mRNA. The presence of three T-DNA copies in plant 103 (data not shown) may explain the highest expression level; the other observed differences may be due to chromosomal location of the T-DNA insertion.

In greenhouse spraying tests transgenic plants expressing various levels of PAT were fully resistant to high doses of Basta® and Herbiace®, the commercial formulations of PPT and bialaphos, respectively. For example, Basta® effectively kills tobacco plants at 2 l/ha while transgenic plants were fully resistant to repeated spraying with 20 l/ha (Figure 2). Normal applications for weed control in agriculture vary from 2.5 to 7.5 l/ha. The fact that treated resistant plants did not show any increase in NH_4^+ content proved the complete protection of the plant GS from the action of the herbicide. Expression of PAT at the level of 0.001% of total extracted proteins is sufficient to protect the plants against field-dose application of the herbicides. Some plants express PAT at a 100-fold higher level. The PPT resistance was inherited in the F1 progeny of tobacco as a single dominant trait. Spraying of the seedlings can be used to follow segregation. Thus, the *bar* gene offers a great advantage for selection of progeny grown in soil; this in contrast with many other marker genes.

Other approaches to obtain PPT-resistant plants have been proposed. Donn *et al.* (1984) selected PPT-tolerant alfalfa cell suspensions. These resulted from gene amplification which yielded an increase in the GS expression. A similar result can likely be obtained by overexpressing a GS gene, using a strong plant promoter. However, it is not clear whether converting more glutamate into glutamine would not produce undesired effects on the nitrogen metabolism of the plant (Loyola-Vargas and Sanchez de Jimenez, 1984). Another approach would be to introduce into plants a mutant form of GS which is no longer inhibited by PPT. Since plant GS can complement a *gln* A mutation in *E. coli* (Das Sarma *et al.*, 1986), it might be possible to select directly in *E. coli* for mutant forms of the plant GS which are resistant to PPT. However, severe drawbacks could be encountered if the resistant GS has modified enzymatic properties. Also, GS has a multimeric subunit structure and mixed enzyme complexes will probably be inhibited by the herbicide. Furthermore, it is

not clear whether overexpression or mutation of a single GS form would result in herbicide resistance since many plants contain multiple forms of GS (Ericson, 1985; Gebhardt *et al.*, 1986).

Our results clearly show that engineering herbicide resistance using a detoxification or degradation process holds much promise. The successful engineering of a detoxification pathway will be largely independent from the plant species used. The same gene will thus be useful to engineer a variety of crops, as we have exemplified here. There are two sources for detoxifying enzymes in nature; some herbicide-tolerant crop species have detoxifying pathways such as the glutathione-S-transferase in corn (Shimabukuro *et al.*, 1971). Another source can be degradative microorganisms often found in herbicide-contaminated soils. Problems with this approach could arise if several enzymes are involved in the detoxification or if the enzymes have only a low affinity and/or specificity for the herbicides. The advantages of PAT as described here are in part the consequence of its evolution as resistance gene to act very efficiently and with high specificity on phosphinothricin.

Materials and methods

Plasmid constructions

All DNA manipulations were as described by Maniatis *et al.* (1982). A *FokI–BglII* fragment was isolated from pBG39 (accompanying paper) and annealed with two complementary synthetic oligonucleotides 5'-CATGAGCCCAGAAC-3' and 5'-CGTCGTTCTGGGCT-3' and ligated to pLKS62 (Botterman, 1986) which had been digested with *NcoI* and *BglII*. The *bar* gene could be isolated as a *BamHI* fragment from the resulting plasmid pGSFR1. A cauliflower mosaic virus 35S promoter fragment, obtained from pGSJ280 (Deblaere *et al.*, 1987) was fused at the *ClaI* site to the *BamHI* fragment containing the *bar* gene, after filling the protruding ends with Klenow DNA polymerase. The TL-DNA gene 7, isolated from pAP2034, was added as the 3'g7 fragment (Velten and Schell, 1985).

A chimeric kanamycin resistance marker containing a nopaline synthase promoter, the neomycin phosphotransferase II gene from Tn5 and the 3' end of the octopine synthase gene was inserted in the *BglII* site of pGV1500 (Deblaere *et al.*, 1987). Subsequently, the chimeric *bar* gene was inserted in this vector to generate pGSFR280.

Transformation of regenerating SR1 tobacco protoplasts by co-cultivation with Agrobacterium tumefaciens

Leaf protoplasts of *N. tabacum* c.v. Petit havana (SR1) (Maliga *et al.*, 1973) were isolated from sterile shoot cultures grown on medium containing half the concentration of MS salts (Murashige and Skoog, 1962), supplemented with 1% sucrose, 0.8% agar, pH 5.7. SR1 tobacco protoplasts were transformed using a modification of the co-cultivation technique of Marton *et al.* (1979). Five ml of protoplasts ($\sim 10^5$/ml) were cultured in K3 medium (Nagy and Maliga, 1976), supplemented with 0.4 M sucrose, 0.5 g/l MES, pH 5.5, 0.1 mg/l naphthaleneacetic acid (NAA) and 0.2 mg/l benzylaminopurine (BAP) in 9 cm Petri dishes for 3 days. Regenerating protoplasts were then infected at a ratio of ~ 100 bacterial cells, grown in Min A medium (Miller, 1972) per protoplast. After 3 days, 2.5 ml of modified K3 medium containing 1.2% low gelling agarose and 1000 mg/l cefotaxime was added to 2.5 ml of the co-cultivation mixture. The culture was incubated in 5 cm Petri dishes. One week later the agarose discs were transferred to 9 cm Petri dishes containing 10 ml of modified K3 medium with 0.35 M sucrose and 50 mg/l kanamycin or 0.5–50 mg/l PPT. Every 5 days the liquid medium was replaced by fresh medium, each time lowering the sucrose concentration by 50 mM. When the sucrose concentration reached 0.1 M, the calli were transferred to B5 medium (Gamborg, 1968) with 250 mg/l NH_4NO_3, 0.5 g/l MES pH 5.7, 0.2 mg/l BAP, 0.6 mg/l NAA, 0.6% agarose and supplemented with various concentrations of kanamycin or PPT.

Leaf disc transformation

Tobacco. Leaves from sterile shoot cultures of *N. tabacum* cv. Petit Havana SR1, grown on medium containing half the concentration of MS salts supplemented with 1% sucrose, 0.8% agar, pH 5.7, were used as starting material. The leaf disc transformation was essentially done as described by Deblaere *et al.* (1987). Midrib and edges were removed from the leaves and segments of ± 0.25 cm^2 were placed in the infection medium (B5 medium supplemented with 250 mg/l NH_4NO_3, 0.5 g/l MES, pH 5.5 and 3% sucrose). To 10 ml of infection medium (in a 9 cm Petri dish) 25 µl of a late log culture of the *Agrobacterium* strain, grown in Min A, was added. After 2 days the leaf discs were washed with the infection medium supplemented with 500 mg/l cefotaxime. The leaf discs were placed on shoot-inducing medium [B5 medium supplemented with 250 mg/l NH_4NO_3, 0.5 g/l MES pH 5.7, 2% glucose, 40 mg/l adenine, 0.8% agar, 1 mg/l BAP, 0.1 mg/l indole acetic acid (IAA) and 500 mg/l cefotaxime] containing the selective agent (PPT or kanamycin). After 3–4 weeks the regenerating calli were transferred to the same medium without IAA and containing 200 mg/cefotaxine. Two to three weeks later, shoots were isolated and transferred to rooting medium (half the concentration of MS salts supplemented with 3% sucrose, 0.5 g/l MES pH 5.7 and 100 mg/l cefotaxime). Roots formed after 1–2 weeks. The rooted shoots were propagated as sterile shoot cultures or transferred to the greenhouse.

Tomato. Sterile shoot cultures of *L. esculentum* cv. *Lukullus*, were grown as described for tobacco. The midrib was removed from the leaves which were cut without wounding the segments of 0.25–1 cm^2. The segments were placed in the infection medium (B5 medium supplemented with 250 mg/l NH_4NO_3, 0.5 g/l MES pH 5.5, 3% sucrose, 40 mg/l adenine, 40 g/l mannitol, 0.5 mg/l transzeatine and 0.01 mg/l IAA). To 10 ml of infection medium (in a 9 cm Petri dish) 20 µl of a late log culture of the *Agrobacterium* strain, grown in Min A, was added. After 2 days the leaf discs were washed with the infection medium supplemented with 500 mg/l cefotaxime. The leaf discs were placed on shoot-inducing medium [B5 medium supplemented with 250 mg/l NH_4NO_3, 0.5 g/l MES pH 5.7, 0.5 g/l polyvinylpyrrolidone (PVP) 200 mg/l glutamine, 2% glucose, 40 mg/l adenine, 40 g/l mannitol, 0.6% agarose, 0.3 mg/l transzeatine, 0.01 mg/l IAA and 500 mg/l cefotaxime] containing 50–100 mg/l kanamycin. Each 5 days the osmotic pressure of the medium was lowered by decreasing the mannitol concentration with 10 g/l. After 1 month calli and meristems were separated from the leaf discs and placed on shoot-inducing medium without mannitol and IAA and containing 1 mg/l transzeatine and 200 mg/l cefotaxime. After small shoots had emerged, the calli were transferred to elongation medium (B5 medium supplemented with 250 mg/l NH_4NO_3, 0.5 g/l MES pH 5.7, 0.5 g/l PVP, 2% glucose, 40 mg/l adenine, 0.6% agarose, 1 mg/l transzeatine and 0.01 mg/l GA_3). The elongated shoots were transferred to medium containing half the concentration of MS salts supplemented with 2% sucrose, 0.5 g/l MES pH 5.7, 100 mg/l cefotamine and 0.7% agar. The rooted shoots were propagated as sterile shoot cultures or transferred to the greenhouse.

Potato. Transgenic potato were also obtained by a leaf disc infection method similar to the one for tobacco. The details of this procedure will be published elsewhere (De Block *et al.*, in preparation).

Herbicide applications

Plants were sprayed with a 2% aqueous solution of the formulated Basta® containing 20% D,L-PPT (Hoechst AG) or Herbiace® containing 33% bialaphos (Meiji Seika, Ltd) from the four sides in a 1 m^2 surface using air-brush line of Badger. Six week old F1 seedlings, planted separately in multijar plates, were sprayed with 8 l Basta/ha.

PAT assays

T.l.c. method. To 100 mg of tissue, 50–100 µl of extraction buffer [50 mM Tris-HCl, pH 7.5, 2 mM Na_2-EDTA, 0.15 mg/ml leupeptine, 0.15 mg/ml phenylmethylsulphonyl fluoride (PMSF), 0.3 mg/ml bovine serum albumin (BSA), 0.3 mg/ml DDT] was added with 5 mg PVP and sea sand. The tissue was ground with a glass rod in a punctured Eppendorf tube that contained a hydrophobic plug at the bottom. The Eppendorf tube was placed into a larger tube and centrifuged for 1 min at 1000 r.p.m. The extract which was recovered was clarified in an Eppendorf centrifuge for 5 min. To a diluted extract (12.5 µl) PPT (0.75 µl of a 1 mM stock in 50 mM Tris pH 7.5, 2 mM Na_2-EDTA) and [^{14}C]AcCoA (1.25 µl) (58.1 mCi/mmol; NEN) were added. The reaction mixture was incubated at 37°C for 30 min and spotted (6 µl) on a silicagel t.l.c. plate. Ascending chromatography was carried out in a 3 to 2 mixture of 1-propanol and NH_4OH (25% NH_3). ^{14}C was visualized by autoradiography (XAR-5-Kodak film overnight).

Spectrophotometric assay. Plant tissue (250 mg) was ground in 500 µl of the half concentrated extraction buffer without DDT. The extract was enriched for the PAT enzyme by a differential $(NH_4)_2SO_4$ precipitation (30–60%). The protein pellet was dissolved in 200 µl of buffer (50 mM Tris-HCl pH 7.5, 2 mM Na_2-EDTA). The protein concentration was measured relative to BSA as standard using the Biorad kit. The rest of the procedure was as described for chloramphenicol acetyl transferase (Shaw, 1975). The rate of PPT acetylation was quantified by measuring the free CoA sulfhydryl group coincident with transfer of the acetyl group to PPT. The reaction of the reduced CoA with 5,5' dithiobis-2-nitrobenzoic acid (DTNB) yields a molar equivalent of free 5-thio-2-nitrobenzoic with a molar extinction coefficient of 13 600 at 412 nm. The net change in extinction per minute was divided by 13.6 to give the result in µmol/min of PPT-dependent DTNB generated. One unit of PAT is defined as 1 µmol of PPT acetylated per min at 37°C.

Isolation of plant RNA and hybridization

Leaf RNA was isolated essentially as described by Jones *et al.* (1985). A

*Bgl*II–*Sal*I fragment from pGSFR280, carrying the *bar* gene, was cloned into the SP6 vector pGEM-2 (Promega-Biotech). SP6 RNA polymerase was used to transcribe a radioactive RNA probe complementary to the *bar* mRNA. Northern hybridization experiments were as described by Jones *et al.* (1985).

Western blot

Aliquots of leaf extracts corresponding to 75 μg total extracted protein were electrophoresed on 12.5% SDS–polyacrylamide gel and transferred to a nitrocellulose filter. The filter was treated first with rabbit antibody to PAT, then with alkaline phosphatase-labelled goat anti-rabbit Ig. BCI8/NBT alkaline phosphatase color development solution (Biorad) was used as substrate.

Ammonia determination in plant extracts

Leaves (250 mg) were extracted as described previously in 0.5 ml extraction buffer containing 0.1 g PVP. Insoluble material was pelleted and 1.6 ml trichloroacetic acid (0.3 M) was added to 0.4 ml supernatant. After 30 min the precipitate was removed by centrifugation. The supernatant was neutralized with KOH (10 mol/l), filtered and used for the determination of NH_4^+ (0.1–0.5 ml). In the presence of glutamate dehydrogenase and reduced nicotinamide-adenine nucleotide (NADH) ammonia reacts with α-ketoglutarate to L-glutamate, and NADH and ammonia are consumed stoichiometrically with the amount of ammonia. The assay was performed in 1 ml cuvettes containing 0.15 M triethanolamine pH 8.6, 11.1 mM α-ketoglutarate, 0.2 mM NADH and 8 U glutamate dehydrogenase. Consumption of NADH was monitored by recording the change in absorbance at 340 nm.

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